Doctoral thesis summary

The role of the non-catalytic domains in the regulation of the C1r serine protease

Author: Balázs Major

Supervisor: Dr. Péter Závodszky
Research Professor, Member of HAS

Institute of Enzymology, Research Centre for Natural Sciences, Hungarian Academy of Sciences

Eötvös Loránd University, Faculty of Science
Biology Doctorate School (Leader: Dr. Anna Erdei)
Stuctural Biochemistry Program (Leader: Dr. László Gráf)

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Introduction

In vertebrates the complement system plays an important role in the innate and acquired host defense mechanisms against infection and in various immunoregulatory processes on both molecular and cellular levels. Three pathways of activate the complement system are known: the classical pathway, the lectin pathway and the alternative pathway.

The classical pathway is triggered by the activation of the C1 complex. This occurs when the C1q binds to IgM or IgG complexed with antigens. Binding to the immunocomplex causes a conformational change in the C1 complex, which leads to activation of the cascade.

The C1 complex, which is a heteropentamer, is composed of one recognition molecule, the C1q, and two molecules of C1r and two molecules of C1s.

Both C1r and C1s zymogen serine proteases consist of six domains. Five domains out of six regulate the proteolytic function of these proteases.

We still do not have a clear picture about the conformation of the C1 complex and the molecular mechanism of its activation. The current models of C1 complex are built from mosaics and the gaps are often filled with speculative elements.

Our knowledge is most limited regarding the functional role of the non-catalytic modules.

The aim of my work is to reveal the function of the non-catalytic domains of C1r. My intention was to refine the model of the C1 complex by clarifying the role of these domains.

A consensus element of all existing models is that significant flexibility of the C1s-C1r-C1r-C1s is required for the activation.

To tackle these problems, I prepared non-catalytic domains and domain pairs of C1r, and studied these conformations and conformational flexibilities.

In addition my aim was the identification of the Ca^{2+} binding site on the C1r molecule and the clarification of the functional and regulational role of Ca^{2+} binding.

Aim

The complement system represents one of the major effector arms of the immune response. As a part of the innate immunity, it provides the first line of defence against invading pathogens, but it plays an important role in developing and modulating the adaptive immune response, as well. The classical pathway is triggered by the activation of the C1 complex. We still do not possess the exact structure description of the C1 complex at atomic level.

C1q is part of the C1 complex, which comprises a single C1q molecule that harbours the C1s-C1r-C1r-C1s heterotetramer zymogens. The current models of the C1 complex are compiled from fragmented of its knowledge structural elements. The refinement of the C1 model was my first goal. There are many unanswered questions in connection with the structure, the interaction and the function of non-catalytic domains.

The aim of my work is to refine the model of the C1 complex by systematically revealing the structure, the interactions and the relevant conformational changes of these non-catalytic domains.

The first task to be solved was the elimination of the technical problems e.g. preparing the non-catalytic domains and domain combinations of the C1r serine protease using techniques of recombinant protein expression and limited proteolysis.

The first obstacle was the marginal stability of some domains. This problem was avoided by designing domain combinations that are able maintain their native or close-to-native structures.

It is known that C1r binds Ca^{2+}, but the localization of the binding site was uncertain.

My aim was to identify the Ca^{2+} binding site on C1r, to measure the K_D of the Ca^{2+} binding and to describe the conformational effect of the binding of Ca^{2+}. In addition I was aimed at nature and extent of conformational changes induced by Ca^{2+}, and to interpret these in relation with conformational flexibility.

Finally, I intended to put the results in a physiological context by examining the Ca^{2+} binding of a native, zymogen C1r, prepared from blood and by refining the structural model of the activation of the C1 complex taking physiological conditions into account.

Results

Expression and renaturation of non-catalytic domains and domain combinations.

The stability of each non-catalytic domain of C1r on its own is marginal. Conformational stability is due to the stabilizing effect of the neighbouring domains. I have designed domain pairs where stability is satisfying and their size is appropriate to enable the identification of the functions of each domain.

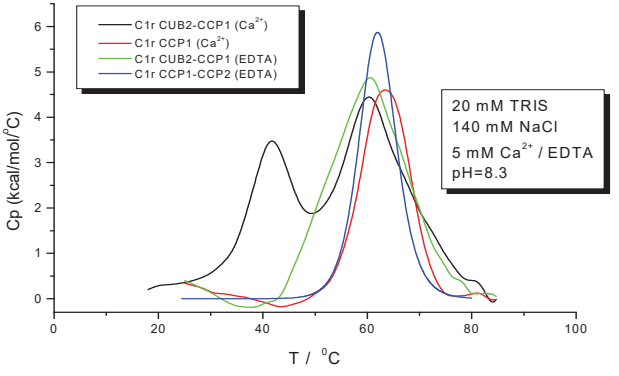
All previous attempts have failed to prepare C1r CCP1. The expression of the C1r CCP1-CCP2 has been successful but its renaturation failed.

The C1r CUB2-CCP1 domain pair was expressed successfully in *E. coli*, renaturated to native structure and the result was confirmed with relevant experiments.

Since the individual expression of the C1r CCP1 domain has never been successful, I prepared it in its native structure by using limited proteolysis from the renaturated C1r CUB2-CCP1 domain pair.

The preparation process of C1r CCP1-CCP2 was similar to the preparation of C1r CCP1: the CUB2 domain was digested from the renaturated C1r CUB2-CCP1-CCP2 construction.

The structures of this domain and the two domain pairs were monitored with circular dichriosm (CD) spectroscopy and differential scanning calorimetry (DSC). Each construction showed its native structure.



Heat denaturation curves of fragments from C1r by DSC.

As a result of attentive preparation and execution, I prepared C1r CUB2-CCP1, C1r CCP1 and C1r CCP1-CCP2 in the quantity of ~20 – 20 mg which is sufficient for functional and structural experiments.

Description the Ca^{2+} binding of C1r CUB2 and determination of the heat stability of this domain.

Microcalorimetry is suitable for the experimental determination of the thermodynamic parameters of protein modules and proteins.

These thermodynamic parameters (T_m, ΔH, C_p) generally give information about conformation stability and flexibility of molecules and the alteration of these features in the case of ligand binding.

The heat denaturation of C1r CUB2-CCP1 shows cooperative transition at 41.5 °C in the presence of Ca^{2+}. It interprets as the melting of the C1r CUB2 domain from the C1r CUB2-CCP1 fragment.

In the absence of Ca^{2+} the C1r CUB2 domain does not show transition. It means that the native fold of C1r CUB2 domain requires Ca^{2+} binding.

Evidence from the results of DSC experiments shows that the C1r CUB2 domain binds Ca^{2+} and the absence of calcium-ion results in a loose structure of C1r CUB2. This way, the Ca^{2+} binding in C1r CUB2 functions as flexibility switch in C1r.

The heat denaturation peak of C1r CCP1 is Ca^{2+}-insensitive, it is sharp and occurs at 63.4 °C. Based on these results, the C1r CCP1 domain could be excluded as a Ca^{2+} binding site. The relatively stable fold of the C1r CCP1 shows that this domain of the C1r is not the source of the flexibility.

Measuring the Ca^{2+} binding affinity of the C1r CUB2 domain and explanation of the relevant role of the unfolded structure.

The Ca^{2+} binding affinity of C1r CUB2 domain was measured by isothermal titration calorimeter (ITC) using the C1r CUB2-CCP1 fragment. I defined the stoichimetric constant as well as the enthalpy change of Ca^{2+} binding.

The K_D value of Ca^{2+} binding of CUB2 domain proved to be 430 ± 20 μM. Using this value I calculated the rate of Ca^{2+} saturation in the C1r CUB2 domain in blood. The 1.2 mM free (ionized) Ca^{2+} concentration observed in the human blood is close to the above binding value, resulting in a theoretical 26% unsaturation of the CUB2 domain.

We compared the enthalpy changes corresponding to the unfolding of CUB2 measured by DSC with that of Ca^{2+} binding measured by ITC. From the DSC, it was possible to calculate the ΔH of unfolding at same temperature, taking into account an average unfolding heat capacity change of a globular protein. The enthalpy change of Ca^{2+} binding indicates that the absence of Ca^{2+} causes significant loss of interactions, i.e. the structure of C1r CUB2 domain is loose whereas it becomes folded upon Ca^{2+} binding.

We concluded that the Ca^{2+} binding of C1r CUB2 domain has relevant regulation function as a switch regulated by the Ca^{2+} concentration of blood.

Structure description of the C1r CUB2 both in the presence and in the absence of Ca^{2+} with circular dichroism (CD) spectroscopy.

The individual C1r CUB2 domain had not been expressed thus I studied its structure in the C1r CUB2-CCP1 domain pair. The secondary structure of C1r CUB2-CCP1 and C1r CCP1 in the presence or absence of Ca^{2+} were characterized with CD spectroscopy. The spectra of the single CCP1 domain, which was proven to be Ca^{2+}-insensitive, the spectral contribution was corrected from spectrums of C1r CUB2-CCP1. In the presence of Ca^{2+}, C1r CUB2 showed a spectrum that is characteristic of proteins containing β-sheet and turn structure, whereas in the absence of Ca^{2+}, the corresponding spectrum was random-like.

Monitoring the conformational dynamics of the C1r CUB2 depending on Ca^{2+} binding by H-D exchange.

The method of monitoring the H-D exchange was used in the case of the C1r CUB2-CCP1 to compare the conformation flexibility both in the absence (unfolded) and in the presence (folded) of Ca^{2+}.

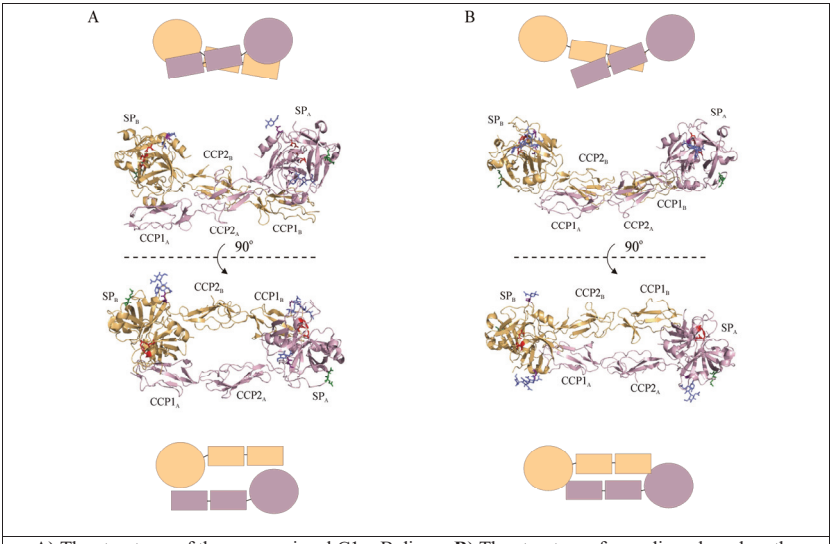
H-D exchange of the Ca^{2+}-free C1r CUB2-CCP1 was fast, indicating that in the absence of Ca^{2+}, the structure of the fragment is loose or unfolded. In the presence of Ca^{2+}, the H-D exchange was one order of magnitude slower, revealing a more compact, folded structure of C1r CUB2-CCP1. The hydrophobic core of the C1r CUB2 evolved only in the presence of Ca^{2+}.

Studying the structure of the C1r CCP1-CCP2 with NMR spectroscopy.

We described the three-dimensional structures of CCP domains and the C1r CCP1-CCP2 domain pair with NMR spectroscopy. The assignation of the domain pair was not trivial but we developed a method through which we carried out these experiments successfully. This way we looked into a specific and relevant aspect of the activation of C1s in the C1 complex.

The zymogen C1r γB fragments show head-to-tail dimer structure in solution, which is stabilized by intermolecular CCP1–SP interactions. We observed interaction between C1r CCP1 and C1r CCP2 domains. This new dimer evolved without the participation of C1r SP. In this case the C1r SP domain can bind to the C1s SP. The conventional C1r dimer is more stable than this CCP1-CCP2 dimer, thus this form has not been detected before. The possible biological significance of this new dimer is the regulation of the activation of the C1s in the C1 complex.

With this result we can explain the delocalization of C1r serine proteases during the activation process of the C1s.



A) The structrure of the convencional C1r γB dimer. **B)** The structure of new dimer based on the interaction of the C1r CCP1-CCP2 domains.

Determination of Ca^{2+} binding affinity of the C1r CUB2 domain on the full-length zymogen C1r from blood and clarifying the role of Ca^{2+} in the autoactivation velocity of the full-length zymogen C1r.

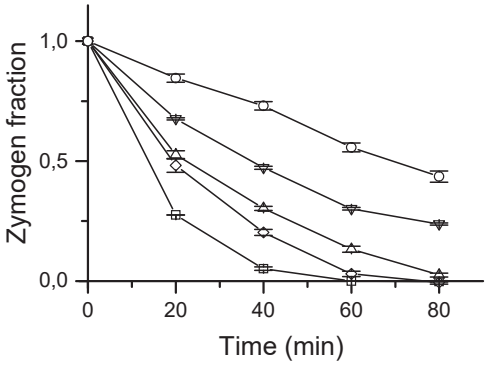
We demonstrated on the renaturated C1r CUB2-CCP1 fragment that the C1r CUB2 domain bears switch function in the autoactivation of the C1r with its flexible structure, marginal stability and low affinity Ca^{2+} binding. To confirm the results that came from the experiments using refolded C1r CUB2-CCP1, I used full-length, zymogen C1r isolated from blood. Full-length C1r has more Ca^{2+} binding sites. Besides CUB2, other sites are probably located at the CUB1 and EGF domains of the C1rα fragment. This fragment binds Ca^{2+} with high affinity (K_D 32 μM). Because the C1rα fragment is already completely saturated at the lowest Ca^{2+} concentration (250 μM) used in this study, the observed effect on autoactivation must be due to the Ca^{2+} binding of the CUB2 domain.

Zymogen C1r was isolated from plasma with specific process. We studied the autoactivation process in the submillimolar- millimolar range. The autoactivation is fast in the absence of Ca^{2+} and the autoactivation velocity decreases with the increase of Ca^{2+} concentration, whereas it is not completed even after 80 min at 1 or 2 mM Ca^{2+}.

In the case of the C1r the velocity of autoactivation was regulated by Ca^{2+} concentration.

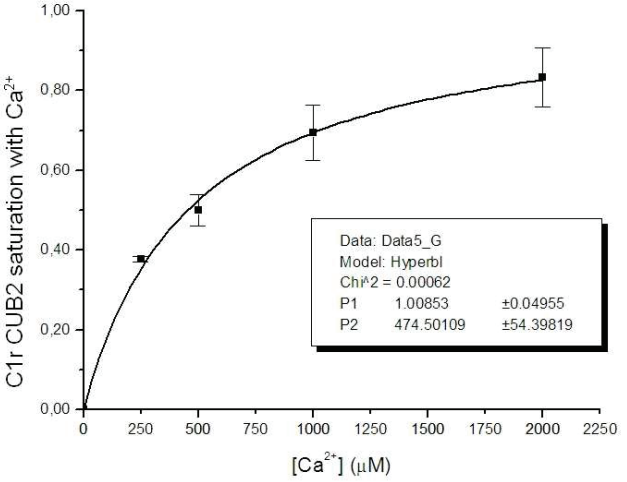
A K_D value of 474 ± 54 μM was estimated from autoactivation rates, which is in good agreement with the K_D value measured for Ca^{2+} binding affinity of the CUB2 domain by ITC (430 ± 20 μM).

Based on our results it is very likely that the CUB2 domain within the refolded C1r CUB2-CCP1 fragment is structurally and functionally equivalent with the CUB2 domain in full-length zymogen C1r from blood. The C1r CUB2 domain regulates the velocity of autoactivation with flexible structure, marginal stability and low affinity Ca^{2+} binding.



The zymogen rate of the C1r on time-dependent curves after normalling and densitomerty. The changing parameter is the Ca^{2+} concentration:100 µM EDTA (□), 250 µM Ca^{2+} (◇), 500 µM Ca^{2+} (△), 1000 µM Ca^{2+} (▽), 2000 µM Ca^{2+} (○).

The C1r Ca^{2+} saturation curve depending on the Ca^{2+} concentration. The fitted hyperbol determine the K_D. This is 475 ± 54 µM. This constant is close to the dissociation constant of the renaturated CUB2 domain with Ca^{2+} by ITC.



Conclusion

The major achievement of my PhD thesis is the refined structural and functional model of the first component of complement (C1); the identification of the Ca^{2+} binding domain (CUB2) and the concept of "flexibility switch" upon Ca^{2+} binding and activation.

The successful preparation of non-catalytic domains and domain pairs from the C1r, using techniques of recombinant protein expression and limited proteolysis opened the way to all of my novel experiments.

This study provides the first direct evidence that the CUB2 domain of C1r binds calcium. It was demonstrated by DSC and ITC measurements. We managed, for the first time, to prove the calcium-binding ability of C1r CUB2 and quantitatively measured its affinity by means of ITC. Ca^{2+} ion induces the folding of C1r CUB2 domain and stabilizes its structure. The C1r CUB2 domain has significantly lower thermal stability than average protein domains (41.5 °C). We revealed that at physiological temperature (37 °C), in the presence of Ca^{2+}, the CUB2 domain is at its border of stability. It is important to note that the melting transition of CUB2 is reversible. The Ca^{2+} ion can serve as a switch between the folded and disordered forms of the C1r CUB2 domain.

We investigated the effect of Ca^{2+} on the autoactivation of the full-length zymogen C1r isolated from blood. The measured K_D value (475 ± 54 μM) estimated from the autoactivation of the full-length zymogen C1r is comparable with the K_D of the renaturated CUB2 Ca^{2+} binding (430 ± 20 μM) determined by ITC. We can conclude that the C1r CUB2 domain operates at the margin of its stability providing flexibility for the C1r dimer and probably for the entire $C1r_2s_2$ tetramer in the blood.

A consensus element of all existing models is that significant flexibility of the $C1r_2s_2$ tetramer is needed for the autoactivation of C1r and the subsequent activation of C1s in the C1 complex. The C1r CUB2 domain is the source of this flexibility. Based on our experimental results we refined the existing C1 model to give a mechanistic explanation for the activation of C1.

List of publications

Publications, which serve basis for the thesis:

1) Major, B., Kardos, J., Kékesi, K. A., Lőrincz, Zs., Závodszky, P. and Gál, P. (2010)
Calcium-dependent conformational flexibility of a CUB domain controls activation of the complement serine protease C1r
J. Biol. Chem. **285,** 11863-11869

2) Láng, A., Szilágyi, K., Major, B., Gál, P., Závodszky, P. and Perczel, A. (2010)
Intermodule cooperativity in the structure and dynamics of consecutive complement control modules in human C1r
FEBS Journal **277**, 3986-3998

3) Láng, A., Major, B., Szilágyi, K., Gáspári, Z., Gál, P., Závodszky, P. and Perczel, A. (2010)
Interaction between separated consecutive complement control modules of human C1r: Implications for dimerization of the full-length protease
FEBS Letters **584**, 4565-4569

Other publication:

4) Dobó, J., Major, B., Kékesi, K. A., Szabó, I., Megyeri, M., Hajela, K., Juhász, G., Závodszky, P. and Gál, P. (2011)
Cleavage of Kininogen and Subsequent Bradykinin Release by the Complement Component: Mannose-Binding Lectin-Associated Serine Protease (MASP)-1
PLoS ONE **6,** e20036